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Anaphase lagging mainly explains chromosomal mosaicism in human preimplantation embryos

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BACKGROUND: Cleavage stage embryos as well as postimplantation embryos have been studied extensively over the years. However, our knowledge with respect to the chromosomal constitution of human embryos at the blastocyst stage is still rudimentary. METHODS: In the present paper, a large series of human blastocysts was examined by means of fluorescent *in situ* hybridization (FISH). RESULTS: It was found that only one in four blastocysts (25%) displayed a normal chromosomal pattern. We defined a group of blastocysts (26%) displaying a simple mosaic chromosome pattern (different cell lines resulting from one chromosomal error), an about equally large group of blastocysts (31%) displaying a complex mosaic chromosome pattern, and a smaller group of blastocysts (11%) showing a chaotic chromosome distribution pattern. Six per cent of all blastocysts analysed could not be assigned one of the previously mentioned chromosomal patterns. CONCLUSION: Anaphase lagging appeared to be the major mechanism through which human embryos acquire a mosaic chromosome pattern during preimplantation development to the blastocyst stage.

Key words: blastocyst/chromosomal mosaicism/fluorescent *in situ* hybridization/human embryos/preimplantation development

Introduction

Dosage imbalance of whole chromosomes is likely to result in inviability (Hassold and Hunt, 2001), yet the human species seems to have found a way to withdraw from this general rule. This intriguing phenomenon has long triggered scientists to study aneuploidy in man.

The search for the origin of chromosomal aneuploidy started in the 1970s when cytogenetic analyses of human oocytes revealed meiotic chromosomal aneuploidy. In the following decade, the advent of IVF allowed the study chromosomal errors occurring at the time of, or shortly after, fertilization, indicating the persistent presence of chromosome-specific aneuploidy (Angell *et al*., 1986; Martin *et al*., 1986; Plachot *et al*., 1987). It was not until the 1990s that the introduction of FISH on single cells of preimplantation embryos resulted in the detecting of postzygotic mosaicism.

Many studies have been performed since, and thus the chromosomal constitution of human embryos at early cleavage stages is at present well documented (Delhanty *et al*., 1993; Coonen *et al*., 1994b; Jamieson *et al*., 1994; Munné *et al*., 1994, 1998; Pellestor *et al*., 1994; Harper *et al*., 1995; Delhanty *et al*., 1997; Laverge *et al*., 1997; Iwarsson *et al*., 1999). To date, chromosomal mosaicism is accepted as a common feature of human embryos. However, the relevance of these findings for the *in vivo* situation remains elusive, since it is unlikely that embryos generated during the course of an artificial reproduction treatment fully represent those originating from natural conception. Moreover, only the 'spare' embryos—neither transferred nor cryopreserved—are available for study.

Our knowledge of the occurrence and frequency of chromosomal aberrations in human blastocysts is even more limited, and in order to gain a better understanding of the processes steering the course of chromosomal mosaicism in the human embryo, it is essential to fill this 'gap'.

In one of the first studies, Benkhalifa *et al*. (1993) only focused on the presence of polyploid cells in (IVF) morulae and blastocysts. More recently, a few papers have been published addressing the chromosomal constitution of human blastocysts as ascertained by means of FISH (Evsikov and Verlinsky, 1998; Magli *et al*., 2000; Ruangvutilert *et al*., 2000; Sandalinas *et al*., 2001; Bielanska *et al*., 2002; Hardarson *et al*., 2003). The general message derived from these studies, although based on small numbers, was that the percentage of embryos exhibiting chromosomal mosaicism increases during *in vitro* preimplantation development to almost 100% at the blastocyst stage, whereas the percentage of abnormal cells per embryo decreases to 22% at the blastocyst stage (Bielanska *et al*., 2002). During development until the blastocyst stage

there is no definite selection against most of the chromosomal abnormalities occurring at cleavage stages (Sandalinas *et al*., 2001). Clouston *et al*. (2002) examined human blastocysts using classical cytogenetic techniques and reported, when compared to cleavage stage embryos, a decrease in embryos revealing a haploid or monosomic chromosomal content as well as some trisomies.

The aim of the underlying study was to clarify the chromosomal evolution leading to the chromosomal patterns displayed at the blastocyst stage; in parallel, to investigate the hypothesis that an increased incidence in chromosomal aberrations accounts for the relatively poor developmental prognosis of ICSI embryos as compared to IVF embryos, as was recently reported (Dumoulin *et al*., 2000).

To our knowledge, this study of 299 blastocysts (149 IVF and 150 ICSI) represents by far the largest group of *in vitro*-grown blastocysts chromosomally studied by means of FISH to date.

Materials and methods

Selected for study were only those embryos that remained after the one(s) with sufficient quality had either been transferred or cryopreserved. Embryos were prepared for FISH analysis using the HCl–Tween 20 method (Coonen *et al*., 1994a).

This spreading method was developed explicitly for embryonic interphase nuclei to be analysed by means of FISH and provides excellent nuclear morphology to ensure a reliable interpretation of (multiple) FISH signals. Due to its large scale, the study took a considerable amount of time and was started in a period in which only a two-colour FISH method was available in our laboratory. FISH analysis is therefore restricted to the sex chromosomes and chromosome 18.

Patients

Embryos described in the present study were obtained from couples seeking infertility treatment at the Academic Hospital Maastricht. Patients' characteristics relevant to the present study (type of infertility, maternal age etc.) are described in detail elsewhere (Dumoulin *et al*., 2000).

IVF/ICSI procedure and embryo culture

The stimulation protocol used has been outlined previously (Land *et al*., 1996) and IVF and ICSI procedures have been published elsewhere (Dumoulin *et al*., 2000). Embryo culture conditions were standardized and performed as described in detail elsewhere (Dumoulin *et al*., 2000). It should be noted that culture media used, human tubal fluid (HTF) (Quinn *et al*. 1985) supplemented with 9% (v/v) PPS or IVF-50™ (Scandinavian IVF Science AB, Sweden), are both of a non-sequential nature and as such less suitable to support viable blastocyst formation *in vitro*. ICSI was performed in case of male subfertility. Individuals presenting with severe oligozoospermia underwent constitutional karyotyping and, if an abnormal karyotype was found, were excluded from treatment. In short, the treatment comprised of a standard long GnRH agonist ovarian stimulation protocol, followed by follicle aspiration and IVF or ICSI. Fertilization was evaluated 18–20 h after insemination or injection.

Human preimplantation embryos

Developmental stage and morphology of all embryos was recorded once daily according to published criteria (Bolton *et al*., 1989). Each embryo received an embryo score, calculated by multiplying the morphological grade by the number of blastomeres (Steer *et al*., 1992). Ultrasound-guided transfer of one or two embryos with the highest embryo score took place in the morning of day 2 or 3 post fertilization. On the third day after oocyte retrieval, cryopreservation of embryos consisting of at least seven cells with equally sized blastomeres and showing <30% anuclear fragments was performed. All surplus embryos developing from normally fertilized (two-pronuclear, 2PN) zygotes and unsuitable for cryopreservation were cultured until day 5 or 6 post fertilization to assess their development *in vitro* (Dumoulin *et al*., 2000). Embryos that had formed blastocyst-like structures, defined as a rim of cells surrounding a cavity of extracellular fluid (so called 'morphological' blastocysts), were spread (see Blastocyst spreading).

The cell number was assessed and, if it was >24, blastocysts were checked for their chromosomal constitution using DNA probes specific for both the sex chromosomes (X,Y) and chromosome 18.

Embryos used in this study were obtained from couples undergoing IVF or ICSI for fertility treatment at the Academic Hospital Maastricht, provided that they had given written informed consent. Investigation of embryos was approved by the Research Ethics Committees of the Academic Hospital Maastricht and Maastricht University.

Blastocyst spreading

Blastocysts were transferred from the culture medium to a droplet of spreading solution containing 0.01 N HCl/0.1% Tween 20 on Super Starfrost Plus slides (Maenzel Glaeser®, Germany) (Coonen *et al*., 1994a). Slides were left to air-dry, washed in phosphate-buffered saline (PBS) and further treated for FISH analysis (see FISH procedure).

Lymphocyte preparation

Interphase nuclei from methanol–acetic acid (3:1)-fixed preparations of lysed peripheral blood cells obtained from healthy male individuals were used to test the efficiency and specificity of DNA probes, probe labelling and FISH procedure. In total, 400 nuclei were counted of which all but one presented with FISH signals. Eight nuclei showed signals other than XY/1818 (2*X/1818, 1*Y/1818, 1*XXY/1818, 2*XY/18, 1*X/18 and 1*XXYY/181(1818).

DNA probes

Three different DNA probes were used to study the chromosomes of interest: (i) PBamX5, alphoid probe (insert size 2.0 kb), specific for the centromeric region of the human X chromosome and directly ratio-labelled with FITC-12-dUTP (Boehringer Mannheim)/rhodamine-4-dUTP (Amersham) (Willard *et al*., 1983); (ii) DYZ3, satellite probe (insert size 2.1 kb), specific for the long arm of the human Y chromosome and directly labelled with rhodamine-4-dUTP (Cooke *et al*., 1982); (iii) L1.84, satellite probe (insert size 0.68 kb), specific for the centromeric region of the human chromosome 18 and directly labelled with FITC-12-dUTP. All probes were labelled by nick-translation, dissolved in hybridization mixture (60% formamide (FA)/2×standard saline citrate (SSC) (pH 7.0) and used in a final concentration of 1–2 ng/µl.

FISH procedure

The FISH pre-treatment procedure was performed as described previously (Coonen *et al*., 1994a) with minor modifications. In summary, after blastocyst spreading and washing of slides in PBS, slides were incubated for 10 min at 37°C with pepsin (Sigma; 100 µg/ml in 0.01 N HCl) to increase the accessibility of nuclei for hybridization to the probes. After incubation, slides were rinsed in

Table I. Mean cell numbers of human blastocysts related to type of assisted fertilization

Cell biological feature	Total	IVF	ICSI
No. of nuclei[a]	50.1 ± 20.1	52.1 ± 1.6	47.8 ± 1.4[b]
No. of nuclei FISH analysed[c]	45.6 ± 18.8	47.6 ± 1.4	43.5 ± 1.4[d]
% FISH analysed[e]	91.0	91.5 ± 0.6	90.4 ± 0.7

[a]Mean number of cells per blastocyst ± SEM.
[b]Significantly different (unpaired Student's *t*-test, $P = 0.045$).
[c]Mean number of cells per blastocyst with analysable FISH results ± SEM.
[d]Significantly different (unpaired Student's *t*-test, $P = 0.046$).
[e]Mean percentage of cells per blastocyst with analysable FISH results ± SEM.

PBS and nuclei were fixed by incubation in 1% paraformaldehyde/0.1 mol/l PBS (pH 7.3) for 5 min at 4°C. Finally, slides were rinsed once more in PBS, dehydrated through an ascending ethanol series and air-dried. Multi-target FISH was performed using the probe cocktail as described under DNA probes. The probe hybridization mixture contained 60% FA/2×SSC/10% dextran sulphate and DNA probes in a concentration of 1–2 ng/µl. Nuclear and probe DNA were heat-denatured simultaneously at 70°C for 3 min. Slides were placed in a moist chamber and hybridization was allowed to take place over a period of 2 h. Post-hybridization washes (all 5 min) were performed at 42°C with 2×SSC/0.05% Tween 20, at 60 °C with 0.1×SSC and finally at room temperature with 4×SSC/0.05% Tween 20. After washing steps, slides were dehydrated through an ascending ethanol series, air-dried and mounted in glycerol containing antifade (Dabco) and 1 µl/ml 4′,6-diaminidino-2-phenylindole (DAPI; Sigma) to counterstain the nuclei. Nuclei were examined using a Leica DMRB microscope equipped with separate filters specific for FITC, rhodamine and DAPI excitation and a triple excitation filter allowing simultaneous visualization of both DNA and all hybridization signals to exclude signal overlap. In order to differentiate FISH errors from true mosaicism, scoring criteria were applied that included signal size and shape, distance between two hybridization signals and signal-to-nucleus size ratio (Munné *et al*., 1998). Signals were recorded and digitized using a CCD camera and software from Applied Imaging.

Statistics

Mean values were analysed using (un)paired Student's *t*-test or analysis of variance (ANOVA), where appropriate. If the ANOVA test showed significant differences, evaluation was performed using the multiple comparisons test according to Tukey. For statistical analysis, data (mean percentages ± SEM) were subjected to arcsine transformation. Assessment of correlations was performed by linear regression analysis. Pearson's correlation coefficient was calculated using the SigmaPlot statistical package (SPSS Inc., USA).

Chromosomal classification of blastocysts

Blastocysts containing a relative majority of disomic nuclei were regarded as chromosomally normal in origin (directly after fertilization) for the chromosomes under study.

Embryos were regarded as chromosomally normal at the blastocyst stage if not more than one cell revealed a non-disomic pattern for the chromosomes under study or, in case of multiple non-disomic cells, if this pattern seemed to have arisen from chromosome doubling for the chromosomes under study. Chromosomal mosaicism was defined as the co-existence of two or more chromosomally distinctive cell populations (and thus two or more distinctive karyotypes) within a single embryo. For any karyotype to be biologically meaningful, it had to be present in at least two cells (ISCN, 1978). We distinguished a simple mosaic chromosome pattern (different cell lines resulting from one chromosomal error), a complex mosaic chromosome pattern (different cell lines resulting from more than one chromosomal error) and a chaotic chromosome distribution pattern (four or more chromosomally unrelated cell lines). The patterns that needed an extraordinary number of chromosomal errors to be explained were defined as unexplained mosaics.

Results

In total, 2915 2PN surplus embryos (1253 IVF and 1662 ICSI) were cultured with patients' permission. Of those, 710 developed to the blastocyst stage and 629 (326 IVF and 303 ICSI) were fixed. Of the blastocysts successfully fixed, 408 blastocysts (218 IVF and 190 ICSI) were analysed by means of FISH. The remaining blastocysts did not meet the minimum cell number criterion. FISH analysis was regarded as informative when ⩾75% of the cells gave interpretable signals. This criterion was met by 299 blastocysts (73%: 149 IVF and 150 ICSI).

Cell biological and chromosomal features of human blastocysts

The mean number of cells per blastocyst amounted to 50 and differed slightly between the IVF and ICSI group (Table I). The use of non-sequential culture media, which is regarded as suboptimal for viable blastocyst formation *in vitro* (Jones *et al*., 1998), and the use of minor quality surplus embryos (non-transferred, non-cryopreserved) may account for this relatively low cell number. The mean percentage of cells per blastocyst that could be analysed by means of FISH was 91% and no differences were noted between the IVF and ICSI group (Table I).

A mean of 72% of all analysable nuclei per blastocyst showed a normal chromosomal content with respect to the sex chromosomes and chromosome 18. No differences were found between either the different types of assisted fertilization or between the different chromosomes investigated (Table II). A slight but significant difference was found for the total group of blastocysts when the mean percentage of nuclei disomic for the sex chromosomes (81.9 ± 0.8) was compared to the mean percentage of nuclei disomic for chromosome 18 (78.3 ± 0.8) (paired Student's *t*-test, $P < 0.001$). As a result, a significant difference was found for the blastocysts analysed when the mean percentage of nuclei monosomic for the sex chromosomes (7.6 ± 0.5) was compared to the mean percentage of nuclei monosomic for chromosome 18 (11.6 ± 0.6) (paired Student's *t*-test, $P < 0.001$).

A mean of 4% of all analysable nuclei per blastocyst showed monosomy with respect to the sex chromosomes and chromosome 18, and 72% and 3% for the proportion of disomic and tetrasomic nuclei respectively. The percentage of nuclei trisomic for the chromosomes tested was negligible in the group of blastocysts analysed. With respect to the above-mentioned parameters, no differences were found between either the different types of assisted fertilization or between the different chromosomes investigated (Table II).

Table II. Chromosomal features of human blastocysts related to type of assisted fertilization

Chromosomal feature	Total group			IVF			ICSI		
	XY,18	XY	18	XY,18	XY	18	XY,18	XY	18
% disomic nuclei[a]	71.7 ± 0.9	81.9 ± 0.8[b]	78.3 ± 0.8	74.3 ± 1.3	83.2 ± 1.1	80.0 ± 1.0	71.6 ± 1.4	81.2 ± 1.2	78.0 ± 1.2
% monosomicnuclei[b,c]	4.2 ± 0.6	7.6 ± 0.5[b]	11.6 ± 0.6	3.0 ± 0.3	6.8 ± 0.7	10.7 ± 0.6	4.1 ± 0.6	8.2 ± 0.9	12.3 ± 1.0
% tetrasomic nuclei[d]	3.3 ± 0.6	5.2 ± 0.5	5.3 ± 0.5	3.2 ± 0.4	5.1 ± 0.6	4.4 ± 0.5	3.6 ± 0.5	5.2 ± 0.6	4.6 ± 0.6

[a]Mean number of disomic nuclei per blastocyst, defined as number of disomic nuclei per blastocyst/number of analysable nuclei per blastocyst ± SEM.
[b]Statistically significant differences between sex chromosomes and chromosome 18 (unpaired Student's *t*-test, $P = 0.001$).
[c]Mean number of monosomic nuclei per blastocyst, defined as number of monosomic nuclei per blastocyst/number of analysable nuclei per blastocyst ± SEM.
[d]Mean number of tetrasomic nuclei per blastocyst, defined as number of tetrasomic nuclei per blastocyst/number of analysable nuclei per blastocyst ± SEM.

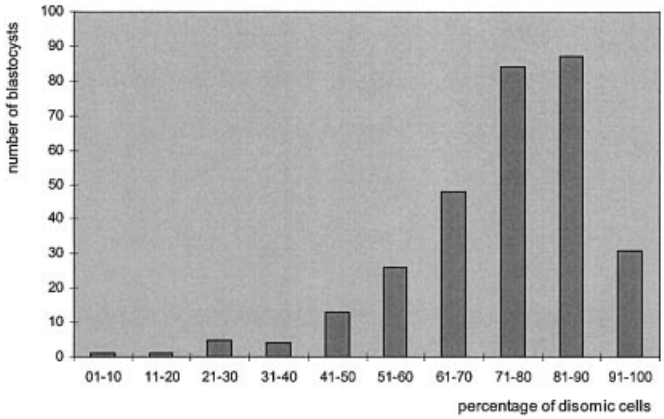

Figure 1. Proportion of disomic cells in human blastocysts.

The sex chromosome distribution among the investigated blastocysts resulted in a 0.93 (XX:XY) sex chromosome ratio. No distortion of sex ratio was noted for the IVF (0.88) or for the ICSI blastocysts (1.00). Based on embryo sex (144 female versus 155 male), no differences were found for any of the investigated chromosomal features shown in Table II. However, the mean number of nuclei per female blastocyst (47.8 ± 1.6) was lower than the one found for male blastocysts (52.3 ± 1.6) (unpaired Student's *t*-test, $P = 0.043$).

All blastocysts analysed were categorized according to their degree of disomy for the chromosomes tested (Figure 1). More than half (57%) of all blastocysts analysed contained a proportion of disomic cells in the range of 70–90%. In all categories, IVF and ICSI embryos were equally represented. No correlation was found between the mean percentage of blastocysts per oocyte retrieval and the mean percentage of disomic nuclei of those blastocysts. Moreover, no correlation was observed between 'cell number per blastocyst' and the percentage of nuclei disomic for the chromosomes under study.

Chromosomal patterns observed in human blastocysts

For each embryo, the chromosomal constitution of every analysable cell was noted. In all but four of the blastocysts, FISH data were not incompatible with a normal chromosomal origin of the chromosomes under study. In 11 out of these 295 blastocysts normal in origin, the cell line disomic for the chromosomes studied showed a relative dominance (highest in absolute number but <50% of all embryonic cells present). In all other cases, the cell line disomic for the chromosomes under study showed an absolute dominance (>50% of all embryonic cells present). There were as many IVF as there were ICSI blastocysts with a normal chromosomal origin.

Those blastocysts not meeting the criteria for normal origin encompassed three blastocysts (two ICSI, one IVF) with a mixture of cells monosomic for the sex chromosomes and chromosome 18 (X18) and cells monosomic for the sex chromosomes (XO,1818) plus a small number of normal cells (XX,1818). The fourth (ICSI) blastocyst abnormal in origin showed monosomy 18 due to meiotic non-disjunction (XX,18). These four blastocysts were not taken into account during further analysis. The majority of blastocysts normal in origin acquired a mosaic chromosome pattern during preimplantation development (Figure 2, Table III). Twenty-five per cent of all blastocysts consisted only of cells normal for the tested chromosomes. This group also includes the blastocysts presenting with cells tetrasomic for all chromosomes investigated. We, as well as others, consider polyploid cells in blastocysts a normal feature of human embryo development (Evsikov and Verlinsky, 1998).

Twenty-six per cent of all blastocysts displayed a simple mosaic chromosome pattern whereas the proportion was 31% for the complex mosaic chromosome pattern.

A smaller group of blastocysts (11%) revealed a chaotic chromosome distribution pattern and 6% of all blastocysts analysed showed an unexplained mosaic pattern.

No differences were found between the different mosaic patterns with respect to the mean number of cells per blastocyst or the mean percentage of chromosomally normal cells per blastocyst. All embryos were checked once daily and received an embryo score based on cell number and cell morphology. No correlation was found between this embryo score on day 2 or day 3 and the type of mosaic pattern observed at the blastocyst stage. IVF and ICSI embryos were represented equally among the chromosomal patterns found at the blastocyst stage. None of the chromosomal patterns displayed a sex ratio that differed from the one observed in the total blastocyst population.

Nature and origin of chromosomal patterns in human blastocysts

Our purpose was to unravel the nature and origin of the chromosomal patterns observed in the blastocysts studied. Therefore, the chromosomal complement (karyotype) of every

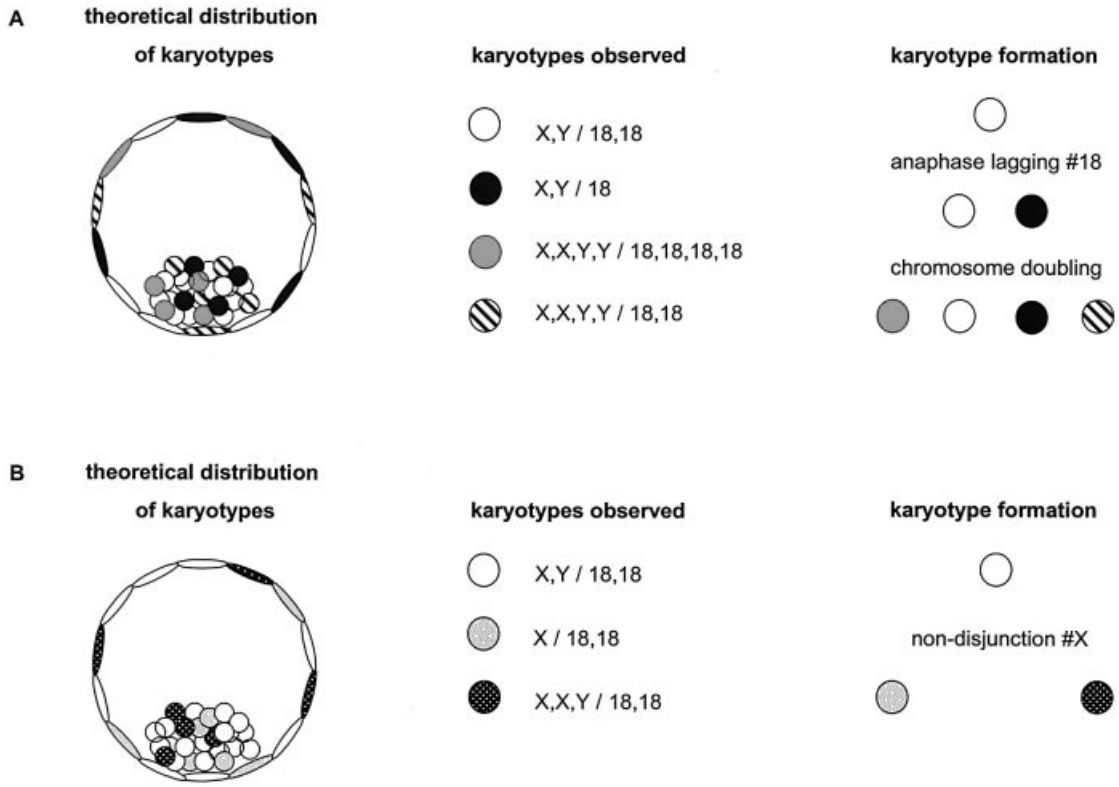

Figure 2. Schematic representation of the aetiology of chromosomal mosaicism observed in blastocysts.

Table III. Chromosomal fate of human preimplantation embryos grown *in vitro* until the blastocyst stage

No. of embryos analysed	299
No. of embryos chromosomally abnormal in origin	4 (1.3)
No. of embryos chromosomally normal in origin	295 (98.7)
No. of embryos chromosomally normal at blastocyst stage	74 (25)
No. of embryos chromosomally abnormal at blastocyst stage	221 (75)
Simple mosaic	77 (26)
Complex mosaic	92 (31)
Chaotic	34 (11)
Unexplained	18 (6)

Values in parentheses are percentages.

analysable cell was noted and the incidence of the various 'karyotypes' was calculated (Table IV). Subsequently, the aetiology of the mosaic pattern was assessed (Figure 2).

Mosaic chromosome patterns observed in blastocysts could originate from a first division failure, leading to a polyploid- or haploid-derived chromosomal pattern but usually seem to originate from errors occurring at the second or later cleavage division giving rise to a diploid-derived chromosomal pattern. Two major pathways were distinguished that contributed to the chromosomal variety seen in mosaic chromosome patterns, namely mitotic non-disjunction and anaphase lagging.

One in three mosaic blastocysts (most frequently complex mosaic embryos) contained cells monosomic for all chromosomes tested (mean incidence 8%), whereas disomic cells were seen in all. Trisomic cells were observed in ~16% of all mosaic blastocysts and tetrasomic cells (indicative of chromosome doubling) in one in four mosaic blastocysts (mean incidence 10%). Two-thirds of all mosaic blastocysts contained cells presenting with monosomy 18, and one in three blastocysts (preferentially complex mosaics) contained cells that showed monosomy for the sex chromosomes. Trisomy for either the sex chromosomes or chromosome 18 occurred less frequently and was mostly observed in chaotic embryos. Cells showing trisomy for the sex chromosomes in combination with monosomy for chromosome 18 (and vice versa) were hardly observed in mosaic blastocysts and seemed restricted to chaotic embryos. Karyotypes that have arisen through chromosome doubling were encountered in all mosaic chromosome patterns but mostly in chaotic embryos.

Given the exact number of cells within a mosaic blastocyst displaying a certain karyotype, we were able to retrace the aetiology of the mosaic pattern (Table V). By definition, the simple mosaic pattern was caused by one division error. Here, the frequency of anaphase lagging was five times higher than that of mitotic non-disjunction. In 78% of the cases, chromosome 18 was affected. Two-thirds of all the complex mosaic blastocysts were abnormal due to anaphase lagging of both an autosome and a sex chromosome. Cells from the remaining blastocysts underwent both mitotic non-disjunction and anaphase lagging with a preference for non-disjunction of the sex chromosomes and anaphase lagging of an autosome. In general, anaphase lagging accounted for 56% of all mosaic patterns observed in human mosaic blastocysts and for 43% of all mosaic patterns seen in the whole group of human embryos grown *in vitro* to the blastocyst stage.

Table IV. Incidence of chromosomal karyotypes in mosaic human blastocysts

Mosaic pattern	Karyotype											
	SS,AA[a]	SA	SSS, AAA	SSSS, AAAA	Poly[b]	SSA	SAA	SS, AAA	SSS, AA	SSS,A	S,AAA	Chromosome doubling[c]
Chaotic (n = 34)												
% embryos displaying karyotype	100	48.5	36.4	54.5	3.0	60.6	27.3	36.4	57.6	24.2	9.1	45.5
Mean % cells[d]		6.4	5.8	12.0	16.0	10.2	5.7	6.3	7.4	5.5	6.0	10.0
Simple mosaic (n = 77)												
% embryos displaying karyotype	100	14.7	5.3	20.0		66.7	14.7	9.3	4.0		1.3	12.0
Mean % cells		7.2	5.0	10.8		14.6	10.5	8.0	4.3		3.0	6.3
Complex mosaic (n = 92)												
% embryos displaying karyotype	100	57.8	13.3	17.8		84.4	62.2	7.8	20.0			10.0
Mean % cells		8.8	8.7	5.3		10.2	7.8	5.0	6.9			6.8
Unexplained (n = 18)												
% embryos displaying karyotype	100		37.5	50.0		31.3	12.5	18.8	12.5	6.3		12.5
Mean % cells			8.0	11.8		8.0	4.5	8.3	7.0	10.0		9.5
Total (n = 221)												
% embryos displaying karyotype	100	37.4	16.1	27.0	0.5	70.6	37.0	13.7	19.9	4.3	1.9	16.6
Mean % cells		8.1	7.5	9.8	16.0	6.8	7.8	6.6	4.7	6.0	5.3	8.2

[a]S = sex chromosome (either X or Y); A = autosome (chromosome 18).
[b]More than four copies of both S and A.
[c]Karyotype indicative of (partial) chromosome doubling (example SSSSAA).
[d]Mean percentage of cells displaying the karyotype within the group of embryos displaying this karyotype.

Table V. Chromosomal patterns in human blastocysts

Subgroup	n	% total	%
Normal (n = 74; 25%)			
No alterations	53	18	73
Chromosome doubling	21	7	27
Simple mosaic (n = 77; 26%)			
Mitotic non-disjunction	14	5	17
S	3		
A	6		
SA[a]	5		
Anaphase lagging	63	22	83
S	12		
A	49		
SA	2		
Complex mosaic (n = 92; 31%)			
Mitotic non-disjunction	4	1	4
S,S[b]			
A,A			
S,A			
SA,S	2		
SA,A	1		
SA,SA	1		
Anaphase lagging	60	21	66
S,S	2		
A,A	1		
S,A	57		
SA,S			
SA,A			
SA,SA			
Both	28	10	31
mndA, alA[c]			
mndS, alS			
mndA, alS	5		
mndS, alA	16		
mndAS, alAS			
mndAS, alA	5		
mndAS, alS	2		
Chaotic (n = 34; 11%)			
Unexplained (n = 18; 6%)			
Total (n = 295)			

[a]S = sex chromosome (either X or Y); A = autosome (chromosome 18).
[b]Two subsequent mitotic non-disjunction events.
[c]mnd = mitotic non-disjunction; al = anaphase lagging.

Patient-related features and chromosomal characteristics of human blastocysts

In 93% of all ICSI treatment cycles, sperm injection was indicated because of male factor subfertility. The two major indications for IVF were tubal pathology (46%) and unexplained subfertility (42%). Based on these three distinctive groups, no differences were found for any of the investigated chromosomal features shown in Table II. Although the chromosomal content of blastocysts did not differ between the three groups, the mean percentage of embryos reaching the blastocyst stage in the male factor group was significantly lower than in the tubal factor and unexplained subfertility group (ANOVA, Tukey, $P < 0.05$). No preference was found for any chromosomal pattern with regard to type of assisted reproductive treatment in general or IVF indication in particular.

Neither type of infertility (primary/secondary) nor IVF/ICSI outcome (pregnant/not pregnant) was related to the chromosomal content of the non-transferred/non-cryopreserved blastocysts or to any particular chromosomal pattern.

No correlation was found between parameter 'maternal age' and the percentage of nuclei with a normal chromosomal content or the various chromosomal patterns observed at the blastocyst stage.

Discussion

Compared to other species, humans display a low fecundity. Of all human conceptions, only ~25% progress successfully to delivery. To a large extent, embryonic death is caused by chromosomal abnormalities that are primarily the result of chromosomal errors encompassing non-disjunction during (female) gamete formation. Some errors will arise at the time of fertilization but most interesting are the recently discovered mitotic errors (non-disjunction or anaphase lagging) that take

place during early cleavage divisions. The latter lead to mosaicism and chaotic chromosome distribution, most probably reflecting asynchrony between karyogenesis and cytokinesis. Selection against embryos showing aneuploid properties (immanent to mosaicism and chaotic patterns) is likely to occur at the time of morula/blastocyst transition. However, our knowledge with respect to the chromosomal constitution of human embryos at the blastocyst stage is still rudimentary.

Several studies on large numbers of cleavage stage embryos have shown that the proportion of chromosomally abnormal embryos varies between 30 and 70%, depending on intrinsic parameters such as embryo morphology, developmental stage, (ab)normal development, presence of multinucleated blastomeres, genetic and/or obstetric background in the case of PGD embryos and technical parameters such as fresh or frozen–thawed embryos and number and type of probes applied simultaneously (Munné *et al*., 1995; Delhanty *et al*., 1997; Laverge *et al*., 1997; Coonen *et al*., 1998; Magli *et al*., 1998; Gianaroli *et al*., 1999; Pellicer *et al*., 1999). Comprehensive chromosomal analysis of single blastomeres of cleavage stage embryos by means of comparative genomic hybridization (CGH) has revealed a proportion of 25% of embryos comprised of normal cells only (Voullaire *et al*., 2000; Wells *et al*., 2000).

We have made a chromosomal inventory of a large group of human blastocysts. It has provided the opportunity to study the evolution of chromosomal abnormalities, leading to the chromosomal patterns found in embryos at blastocyst stages. It should, however, be kept in mind that the embryos studied have been cultured *in vitro*. It is as yet unclear to what extent this *in vitro* environment may affect the (chromosomal) development of a human embryo. Moreover, embryos have been highly selected and may not truly reflect the general blastocyst population, as the embryos of best quality (according to morphological criteria) have been selected and used for either fresh transfer or cryopreservation. Furthermore, only a limited number of chromosomes was investigated.

Chromosomal status of human blastocysts

We have shown that the vast majority of human blastocysts studied displays a mosaic chromosomal profile and that the extent of mosaicism ranges widely. The karyotype showing disomy for the sex chromosomes in combination with monosomy for chromosome 18 was the most frequent abnormality. With respect to complete chromosome 18 aneuploidy, MII non-disjunction predominates among the possible causes (Hassold and Hunt, 2001). However, trisomy for chromosome 18 was seldom found in the blastocysts studied.

FISH control experiments using the DNA probes specific for the sex chromosomes and chromosome 18 on lymphocytes revealed identical percentages of monosomy, disomy, trisomy and tetrasomy for each individual probe. The slight difference (in absolute numbers) that was found for the total group of blastocysts when the mean percentage of nuclei disomic for the sex chromosomes was compared to the mean percentage of nuclei disomic for chromosome 18 was considered clinically irrelevant. The statistically significant difference found with respect to the mean percentage of nuclei monosomic for the sex chromosomes compared to chromosome 18 could either imply that the number of signals representing chromosome 18 is underestimated as a result of misinterpretation or FISH artefact, or that chromosome 18 is more prone to monosomy than are the sex chromosomes. Studies on embryos using repeated FISH with the same DNA probe specific for chromosome 18 indicate that the first scenario is not likely to be the case (unpublished results).

By definition, the blastocysts displaying a chaotic chromosome pattern contained a large variety of chromosomally abnormal cells. The simple mosaic chromosome pattern was mostly caused by anaphase lagging of chromosome 18. The complex mosaic chromosome pattern encompassed karyotypes, almost exclusively caused by anaphase lagging of one of the sex chromosomes followed or accompanied by anaphase lagging of chromosome 18. It might well be that in embryos with unexplained mosaicism, mitotic non-disjunction had occurred but that the chromosomal counterparts of the karyotypes present had been eliminated from the embryo or were present in the non-analysable cells.

Comparison of cleavage stage embryos and blastocysts with respect to chromosomal status

If the chromosomal status of 'good quality' cleavage stage embryos (believed to have the highest developmental potential) is compared to that of blastocysts (that have actually developed), it is evident that the proportion of embryos comprised only of normal cells decreases dramatically: from ~70% of monospermic, normally developing day 2/3 embryos (Munné *et al*., 1995) to only 25% of the blastocysts studied in this paper. It should be noted that CGH studies revealed only one in four cleavage stage embryos to be comprised of normal cells only. However, larger CGH studies need to be performed in order to establish whether this proportion is a realistic one.

A comparison with FISH data on blastocysts presented by others is hampered due to differences in experimental design and chromosomal classification, amongst which the definition of 'chromosomally normal'. Magli *et al*. (2000) as well as Sandalinas *et al*. (2001) studied blastocysts that developed from chromosomally abnormal (determined by means of FISH) day 3 embryos. Proportions of 0% (0/16) (Magli *et al*., 2000) and 39% (21/54) (Sandalinas *et al*., 2001) blastocysts with a normal chromosomal constitution were reported. In the paper of Magli *et al*. (2000) no definition of chromosomally normal was provided (only ICM cells were analysed by means of FISH) and Sandalinas *et al*. (2001) defined chromosomally normal as <10% of the analysed cells being abnormal, including in the chromosomally normal group also the blastocysts that contained <38% tetraploid cells. Ruangvutilert *et al*. (2000), Bielanska *et al*. (2002) and Hardarson *et al*. (2003) studied blastocysts developing from a non-transferred, non-cryopreserved embryo population, reporting proportions of 11% (2/19), 9% (3/33) and 42% (19/45) respectively of blastocysts with a normal chromosomal constitution. Both Ruangvutilert *et al*. (2000) and Hardarson *et al*. (2003) applied a threshold percentage of 10% abnormal cells in order to define chromosomally normal blastocysts, but unlike Sandalinas *et al*. (2001) blastocysts containing tetraploid cells were not included. In the study of Hardarson *et al*. (2003) 16%

of analysed 'surplus' blastocysts presented with a disomic/tetrasomic mosaicism, comprising 27% of all mosaic patterns found. Bielanska *et al*. (2002) defined embryos as chromosomally normal if—compared to control lymphocytes—in an equal or lower percentage of cells a chromosomal abnormality was present. Blastocysts presenting with tetraploid cells were regarded as mosaic and comprised 77% of all mosaic patterns found.

Different types and varying degrees of chromosomal mosaicism have thus been demonstrated in human blastocysts, as has been the case for cleavage stage embryos.

In contrast to the chromosomal abnormalities seen in cleavage stage embryos, mosaic chromosome patterns observed in all but one blastocyst included in our FISH analysis had originated from mitotic division errors. A meiotic division error would render all embryonic cells chromosomally aneuploid. Also others have shown no or only few blastocysts presenting with aneuploid cells only (Magli *et al*., 2000; Ruangvutilert *et al*., 2000; Sandalinas *et al*., 2001; Bielanska *et al*., 2002; Hardarson *et al*., 2003). Our study shows that severe chromosomal abnormalities such as the complex mosaic and chaotic chromosome distribution patterns are less prominent in blastocysts than in cleavage stage embryos but do not seem to fully prevent blastocyst formation. These findings are in alignment with data reported by others (Magli *et al*., 2000; Ruangvutilert *et al*., 2000; Sandalinas *et al*., 2001; Bielanska *et al*., 2002; Hardarson *et al*., 2003). Hence, the presumption of selection against aneuploid embryos occurring at the time of morula/blastocyst transition (Evsikov and Verlinsky, 1998) is only partially supported.

Comparison of IVF and ICSI blastocysts with respect to chromosomal status

The data generated allowed us not only to analyse human blastocysts as such but also to compare blastocysts originating from IVF or ICSI. It should be noted that, as far as oocyte maturity was concerned, we did not separate the *in vitro*-maturated (within 3 h after oocyte retrieval) MI–MII oocytes from those that were already MII stage at the time of oocyte retrieval when fertilization was performed by ICSI. When fertilized by means of IVF, no information was available on oocyte maturity.

Despite its success and widespread application, the ICSI technique is still regarded as potentially harmful to the embryo that emerges from it and hence one cannot exclude that subsequent embryo development will be impaired (Edwards, 1999). Possible causative factors might be that sperm used for injection are selected from a population which is at increased risk of chromosomal abnormalities (Moosani *et al*., 1995; Pang *et al*., 1999) as well as the fact that the injection procedure itself may disturb chromosome alignment in the (fertilized) oocyte (Macas *et al*., 1996; Rosenbusch and Sterzik, 1996; Moomjy *et al*., 1999; Dumoulin *et al*., 2001). We demonstrated impaired *in vitro* blastocyst formation after ICSI as compared to the IVF technique (Dumoulin *et al*., 2000).

Although ICSI embryos did slightly worse than IVF embryos with respect to most parameters investigated, differences were not statistically significant. Hence the apparent discrepancy between IVF and ICSI embryos in terms of *in vitro* blastocyst formation cannot be explained by variations in the incidence or the type of chromosomal errors for the chromosomes under study. It seems as though the *in vitro* developmental potential of human IVF and ICSI embryos is tuned by other, subtle (as yet unidentified) conditions. These could include internal parameters (i.e. apoptosis, necrosis, and cleavage arrest) as well as external circumstances [i.e. assisted reproductive treatment indication (maternal/paternal) or the ICSI technique *per se*].

A sizeable proportion of the blastocysts contained between 10 and 30% abnormal cells. This probably indicates that the presence of a relatively small number of non-disomic cells does not cause dysfunction of the embryo *per se*. We postulate that non-disomic cells are a normal feature of human blastocysts as we are able to study them (*in vitro*-grown, surplus embryos) and, provided this proportion stays within certain limits and/or the embryo is capable of eliminating chromosomally aberrant cells in due time, that they do not affect embryo viability and developmental competence. In this respect one could speculate on the appropriateness of extensive aneuploidy screening or CGH to select those embryos having the highest developmental potency. There exists an imaginable risk that, because of the technical complexity of the analysis procedure itself as well as the interpretation of the data gathered, far too many embryos will be classified unsuitable for transfer. From a chromosomal point of view, fine-tuning of the criteria to select for the one embryo superior in every respect, will be one of the major challenges for the near future.

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